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Further evidence of the usefulness of bile acids as molecules for shuttling cytostatic drugs toward liver tumors

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Background/Aims: **To use bile acids as shuttles for directing cytostatic drugs toward liver tumors, the ability of the tumor to take up these compounds must be maintained. Thus, we investigated whether glycocholate (GC) derivatives such as the fluorescent FITC-GC and the cytostatic Bamet-R2 are taken up by neoplastic tissue at different stages of chemically-induced rat liver carcinogenesis.**

Methods: **Placental glutathione-S-transferase (GST-P) was immunohistochemically detected. Uptake studies were carried out on pure GST-P-positive cell cultures, obtained by treatment with ethacrinic acid. FITC-GC, Bamet-R2 or cisplatin was administered (i.v.) to anaesthetized rats. Platinum in culture cells, liver and kidney was measured by flameless atomic absorption.**

Results: **Co-localization after FITC-GC i.v. administration revealed that only 15% (20 weeks) and 30% (32 weeks) of GST-P-positive tissue was not able to take up FITC-GC. GC uptake was lower in GST-P-positive cells than in normal hepatocytes. Bamet-R2, uptake was lower than that for GC, but similar in both cell types. The amount of Bamet-R2 or cisplatin retained by GST-P-positive tissue after *in vivo* administration was progressively increased during carcinogenesis. Moreover, this amount was higher for Bamet-R2 than for cisplatin. By contrast, in the kidney, it was higher for cisplatin than for Bamet-R2.**

Conclusion: **These results indicate that at the different stages of rat hepatocarcinogenesis most GST-P-positive tissue is able to take up bile acid derivatives, such as Bamet-R2.**

Key words: **Cancer; Cisplatin; Ethacrinic acid; FITC-GC; Glutathione-S-transferase; Glycocholate; Hepatocyte transport; Uptake.**

THE EFFICIENCY of bile acid uptake by hepatocytes (1) has led to the suggestion that these steroids could be used as carrier molecules to shuttle drugs to the liver (2–5). Different and important medical applications of this strategy have recently been reviewed (6). Some criticism has been offered with regard to the usefulness of the strategy in the case of cytostatic compounds (7), because it has been reported that cell lines derived from liver tumor cells fail to take up bile acids. Indeed, it has been reported that Na^+-dependent bile acid uptake is lost to a certain extent in rat hepatoma cells (8–11). However, some degree of Na^+-independent uptake is still present in cell lines derived from liver tumors (11,12), thus accounting for efficient bile acid uptake, although it is lower than that of hepatocytes (11,13). Recently, molecular evidence has been reported for the expression of both OATP and NTCP on the surface of human hepatocellular carcinoma cells (14).

Platinum-based chemotherapy, mainly using cisplatin, has been shown to be efficient against a variety of solid tumors (15). However, its dose-limiting toxicity has encouraged the search for new cisplatin derivatives. Our group has synthesized a new antitumoral compound (Bamet-R2) by binding cisplatin to the carboxylate group of glycocholic acid (GC) (16). The remaining binding position of platinum is occupied by Cl^-, which behaves as a leaving group within the cell, where chloride concentrations are lower than in serum. Previous studies have shown that Bamet-R2 binds to DNA and efficiently inhibits tumor cell growth *in vitro* and when subcutaneously implanted in nude mice, where tumor cells are directly exposed to this compound (17). However, the usefulness of Bamet-R2 and

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related compounds depends on their ability to enter liver tumor cells and accumulate in this tissue to reach therapeutic concentrations following oral or i.v. administration; to date, their ability to achieve this aim is not known.

It has been reported that in healthy rats Bamet-R2 behaves as a cholephilic compound (18). The liver uptake and bile secretion of Bamet-R2 are markedly higher than those of cisplatin but lower than those of the parent bile acid, GC. The mechanisms responsible for Bamet-R2 transport across the hepatocyte have been partly characterized. Bamet-R2 uptake is mainly via a Na^+-independent pathway, which is strongly inhibited by cholic acid, and less efficiently so by GC, ouabain and estrone sulfate. Moreover, Bamet-R2 output into bile is highly sensitive to GC inhibition (18). These findings suggest that Bamet-R2 shares part of the hepatocyte machinery involved in bile acid transfer from blood to bile. Whether these processes are modified in liver tumors and are able to reduce or enhance accumulation of these complexes in tumoral tissue is not known.

The aims of the present work were therefore to gain information about the ability of *in situ* liver tumors to take up and retain bile acid derivatives, and to evaluate the tissue concentrations of Bamet-R2 in the livers and kidneys of rats with chemically-induced liver carcinogenesis using the Solt & Farber method (19). This consists of a single carcinogenic dose of diethylnitrosamine (DEN), followed by short exposure to 2-acetamidofluorene (2-AAF) sufficient to suppress growth of virtually all normal hepatocytes, and partial hepatectomy to activate rapid growth of DEN-altered hepatocytes not suppressed by 2-AAF.

Materials and Methods

Chemicals and animals

Cisplatin, sodium glycocholate (GC), fluorescein isothiocyanate (FITC) and chemicals used for isolation and culture of rat liver cells were purchased from Sigma Chemical Co. (St Louis, MO, USA). Cholylglycylamido-fluorescein (FITC-GC) was synthesized by binding FITC to GC at the carboxyl group (20) using the method of Sherman & Fisher (21). Bamet-R2 (cis-diamminechlorocholylglycinateplatinum(II)) was synthesized and chemically characterized as previously reported (16). [^{14}C]-GC (specific radioactivity 46.7 mCi/mmol) was obtained from New England Nuclear (Itisa, Madrid, Spain). All other reagents were from Merck (Darmstadt, Germany).

Male Wistar rats were obtained at 6 weeks of age from the Animal House at the University of Salamanca, Spain. They were fed with commercial pelleted rat food (Panlab, Madrid, Spain) and water *ad libitum*. Temperature (20°C) and the light/dark cycle (12 h:12 h) in the room were controlled. All animals received humane care as outlined in the "Guide for the Care and Use of Laboratory Animals" (National Institute of Health Publication No. 80–23, revised 1985).

Animals were randomly divided into 4 groups. The control group received no treatment and experiments were carried out at 8 weeks of age. Liver cancer was induced by a modification of the classical method of Solt & Farber (19) and experiments were carried out at 12, 20 and 32 weeks after initiating the treatment. This consisted of diethylnitrosamine (DEN) i.p. administration (20 mg/100 g body weight) on day 1. From days 14 to 35 they received 2-acetamidofluorene (5 mg/100 body weight, subcutaneously twice a week). On day 21 the animals underwent two-thirds partial hepatectomy (22).

Primary culture of rat liver cells

Liver cells were isolated from control or treated animals by a modification of the procedure of Berry & Friend (23) and suspended in medium A (William's medium E supplemented with 26.2 mM $NaHCO_3$, 10 mM Hepes, 100 nM Na_2SeO_3, 30 nM dexamethasone, 100 nM insulin, 11.1 mM galactose and antibiotics (streptomycin 0.02 mg/ml, penicillin 20 U/ml and amphotericin B 0.05 μg/ml), pH 7.40). Preparations whose viability, as assessed by the trypan blue exclusion test, was lower than 85% were discarded. Hepatocytes from healthy rats or GST-P-positive cells from DEN-treated animals after hepatocyte elimination by incubation for 48 h with different concentrations of ethacrinic acid (24) were seeded (8×10^5 cells/dish) on 3-cm diameter culture dishes previously coated with a layer of type I collagen obtained from rat tail tendons. A 90-min period was allowed before the attachment medium was replaced by culture medium B (medium A supplemented with 1 μM ethanolamine, 10 mM nicotinamide, 6 mM ornithine, 5 nM EGF, 5 μg/ml transferrin, 5 μg/ml linoleic acid, 0.5 mg/ml fatty acid-free albumin). [^{14}C]-GC and Bamet-R2 uptake by liver cells was studied at 24 h of culture by an adaptation (25) of the method described by Liang et al. (26), using an incubation time (5 min) and a substrate concentration (100 μM) appropriate for comparing the overall efficiency of these processes in both cell types.

In vivo *experiments*

After anesthetization of fasting rats by i.p. administration (5 mg/100 g body weight) of sodium pentobarbital (Nembutal N.R., Abbot, Madrid, Spain), a catheter was implanted into the left jugular vein and the common bile duct was cannulated. After a resting period of 15 min, bile was collected for 30 min to measure non-stimulated bile flow. In some experiments FITC-GC was infused i.v. at 100 nmol/min for 30 min. Three 10-min bile samples were collected. At the end of this period, the liver was rapidly washed through the portal vein with 50 ml of 150 mM NaCl followed by 200 ml fixation solution (15% picric acid, 4% paraformaldehyde and 0.08% glutaraldehyde in 0.1 M phosphate buffer at pH 7.3). The liver was cut into small blocks, which were cryo-preserved in 30% sucrose and frozen embedded in Cryo-M-Bed (Bright Instruments, Huntingdon, UK). Thirty-micrometer slices were cut on a Bright Instrument cryostat and serial slices were used for fluorescent microscopy observation and for immunohistochemical study. The number of areas of GST-P-positive tissue in the sections examined was measured using a video image processor (MIP3, MICROM S.A., Barcelona, Spain). The size of GST-P-positive regions was calculated assuming a statistical distribution throughout the liver and a spherical shape of the islands (27). In a separate set of experiments, rats received an i.v. bolus of 250 nmol Bamet-R2 or cisplatin. Bile was collected for 15 min; then, the liver and both kidneys were removed, weighed, homogenized and digested.

Analytical methods and statistical analysis

To carry out the immunohistochemical study, liver slices were incubated with monoclonal antibodies for glutathione-S-transferase-P (previously obtained in mice in collaboration with Dr. A. Toraño and Dr. M. Dominguez at the Instituto de Salud Carlos III, Majadahonda, Madrid, Spain). Biotinylated anti-mouse IgG was used as secondary antibody. The avidin-horseradish peroxidase system (Vectastain ABC detection kit, Vector Laboratories, Burlingame, CA, USA) was used to stain GST-P-positive areas of liver parenchyma. Bile flow was determined gravimetrically assuming 1 μl=1 mg. After digesting the samples in nitric acid, the platinum contents in culture cells, bile, liver and kidney were measured by flameless atomic absorption spectrophotometry using a Z-8100 Polarized Zeeman apparatus with a graphite furnace (Hitachi, Pacisa, Madrid, Spain). Radioactivity due to [^{14}C]-GC was measured in a liquid scintillation spectrometer (LS-1800-Beckman, Beckman Instruments, Madrid, Spain) using the

TABLE 1

Anatomical observations

	Control	Hepatocarcinogenesis		
		12 weeks	20 weeks	32 weeks
Body weight (g)	258±21	368±13[a]	440±36[a]	519±21[a]
Kidney weight (g)	0.94±0.06	1.02±0.05[a]	1.24±0.08[a]	1.46±0.05[a]
Liver weight (g)	6.8±0.4	10.2±0.5[a]	12.6±1.4[a]	15.2±2.1[a]
Liver GST-P(+) tissue (%)	1.0±0.1	31.7±9.1[a]	57.1±1.0[a]	22.3±2.7[a]
Liver parenchyma histology	Normal	Foci	Adenomas	Carcinoma

Values are means±S.E. from experiments carried out in control rats and at 12, 20 and 32 weeks after the initiation of hepatocarcinogenesis (in all groups $n=10$).

[a] $p<0.05$ as compared with the Control group by the Bonferroni method of multiple range testing.

Ready-Safe Scintillation Cocktail, also from Beckman, as scintillant. FITC-GC concentrations in bile samples were measured fluorometrically (Fluorescence Spectrophotometer F-4010, Hitachi). The viability of cells in culture was determined by their ability to take up neutral red (28). Protein was determined by a modification of the classical method of Lowry et al. (29) with bovine serum albumin as standard.

Results are expressed as means±S.E. To calculate the statistical significance of differences among groups, paired and unpaired *t*-tests and the Bonferroni method of multiple-range testing were used, as appropriate. Statistical analyses were performed on a Macintosh LC-III computer (Apple Computer, Inc., Cupertino, CA, USA).

Results

Liver carcinogenesis in rats induced chemically by the Solt & Farber protocol is a well-known model in which several stages of tumor development can be identified by standard criteria (30). Initiated enzyme-altered foci expressing the major placental isoenzyme of glutathione-S-transferase, i.e., GST-P, increased to approximately one third of the liver parenchyma by the end of week 12. This was followed by a hyperplastic stage leading to the formation of fibrous encapsulated adenomas, which occupied approximately half of the liver parenchyma at 20 weeks. Thereafter, GST-P-positive tissue was reduced to one fifth of the liver mass, and transformation into carcinomatous tissue growing outside the dysplastic nodules was seen by week 32 (Table

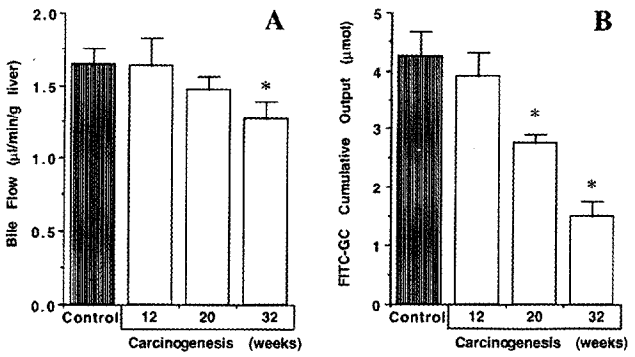

*Fig. 1. Non-stimulated bile flow (A) and cumulative FITC-GC output into bile (B) during a 30-min i.v. infusion of this fluorescent bile acid derivative at 100 nmol/min in Control rats and at 12, 20 and 32 weeks after the initiation of hepatocarcinogenesis. Values are means±S.E. (in all groups n=5). *p<0.05 as compared with the Control group by the Bonferroni method of multiple range testing.*

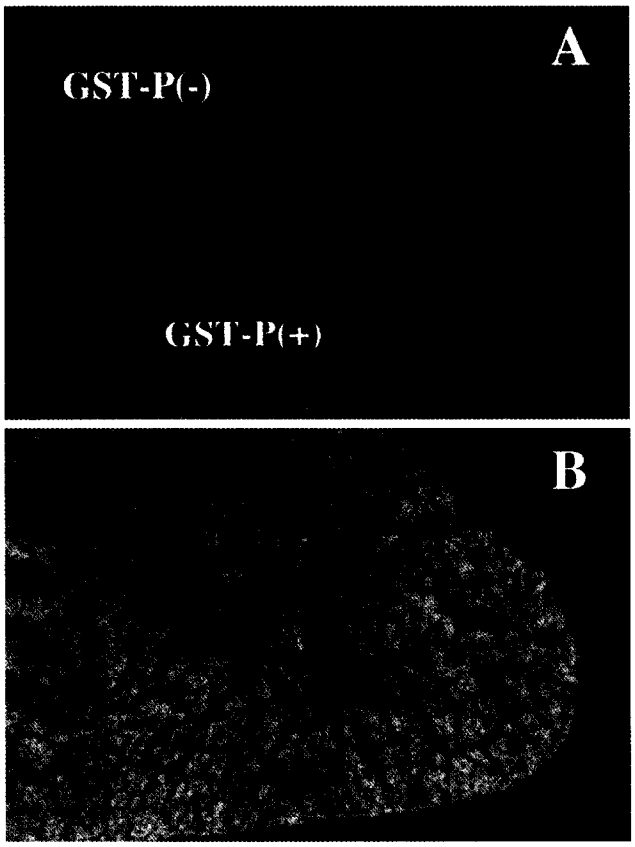

Fig. 2. (A) Immunohistochemical staining with monoclonal anti-glutathione-S-transferase-P (anti-GST-P) antibodies of a 30-µm-thick rat liver slice obtained at 12 weeks after initiating hepatocarcinogenesis. (B) Fluorescence microscopy image obtained from a serial slice of the same liver after receiving 100 nmol/min FITC-GC for 30 min. Both GST-P positive and -negative regions were fluorescent.

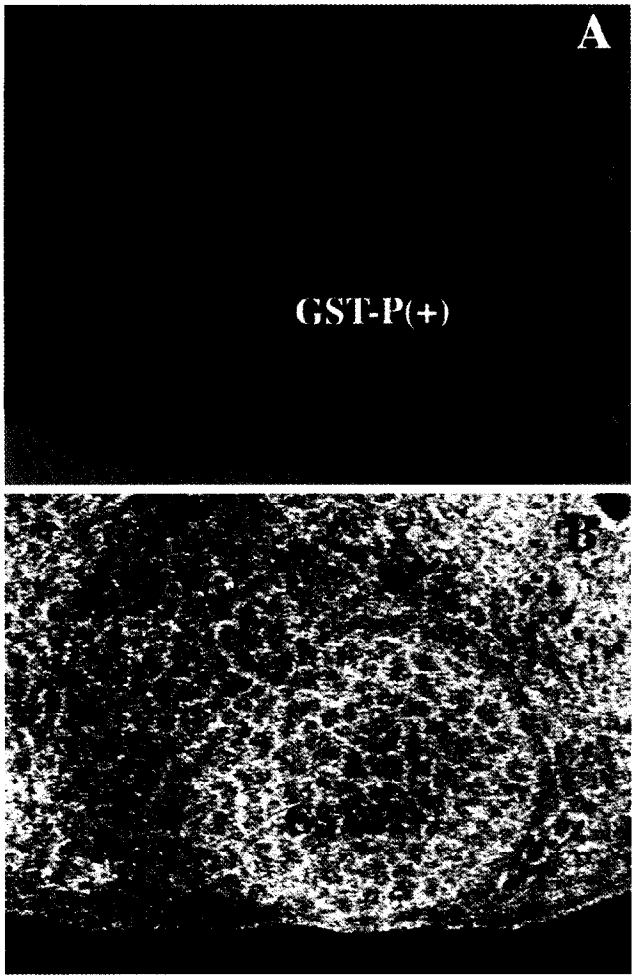

Fig. 3. (A) Immunohistochemical staining with monoclonal anti-glutathione-S-transferase-P (anti-GST-P) antibodies of a 30-μm-thick rat liver slice obtained at 32 weeks after initiating hepatocarcinogenesis. (B) Fluorescence microscopy image from a serial slice of the same liver after receiving 100 nmol/min FITC-GC for 30 min. Both GST-P-positive and -negative regions were fluorescent.

1). Because these lesions took a long time to form, a marked increase in body weight, and proportionally in liver and kidney weight, was observed (Table 1). However, the liver-to-body weight and kidney-to-body weight ratios were unchanged. In all groups, these values remained close to 2.9% and 0.28%, respectively. Kidney metastasis was not observed in any of the animals (data not shown). During liver carcinogenesis, a progressive impairment in biliary function was observed. Non-stimulated bile flow was moderately but significantly reduced (−25%) up to 32 weeks after DEN administration (Fig. 1A). Additionally, following intravenous administration of 3 μmol FITC-GC, this was secreted into bile with lower efficiency as carcinogenesis progressed. Cumulative FITC-GC output into bile was significantly reduced (−33%) as from 20 weeks and was only 36% that of the Controls at 32 weeks (Fig. 1B). Co-localization of FITC-GC loaded cells and GST-P expression in serial liver slices obtained at 12 weeks after DEN-administration revealed that liver tissue expressing GST-P (Fig. 2A) was able to take up FITC-GC (Fig. 2B). Similar results were obtained when FITC-GC was administered to rats 20 or 32 weeks after DEN-administration. Most adenoma and carcinoma tissue, but not the surrounding fibrous capsules were loaded with fluorescence. Fig. 3 shows a carcinomatous nodule showing strong atypia, immunostaining for GST-P (Fig. 3A) and a marked ability to take up FITC-GC (Fig. 3B). However, the co-existence was noted in the same liver of GST-P-positive regions with and without the ability to take up FITC-GC. GST-P-positive tissue lacking fluorescence was 15% at 20 weeks (Fig. 4A) and 30% at 32 weeks (Fig. 4B).

The ability of GST-P-positive cells to take up GC and the cytostatic bile acid derivative Bamet-R2 was investigated *in vitro*. GST-P-positive cells from rat livers

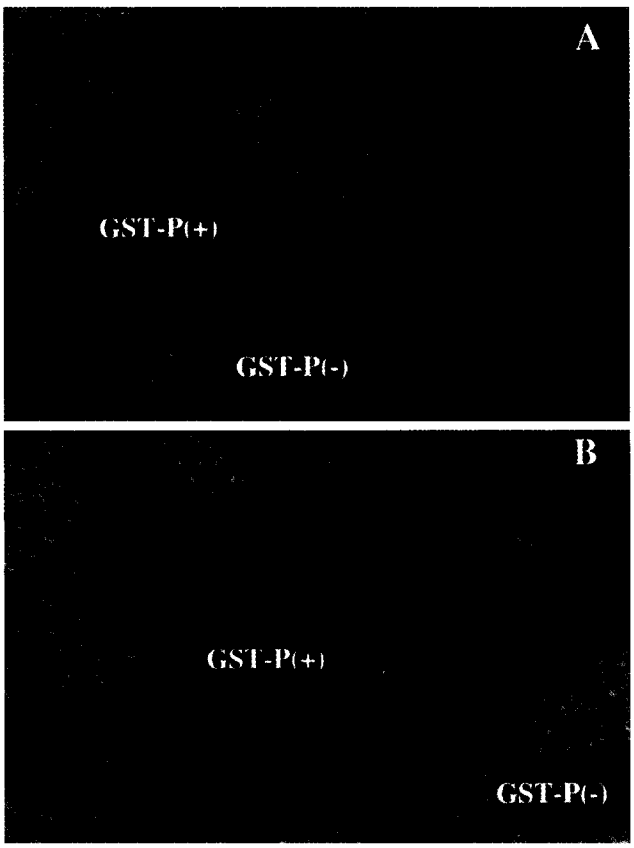

Fig. 4. Fluorescence microscopy images obtained from slices of rat liver after receiving 100 nmol/min FITC-GC for 30 min at 20 weeks (A) or 32 weeks (B) after the initiation of carcinogenesis. GST-P-positive regions appear free of fluorescence while GST-P negative are fluorescent.

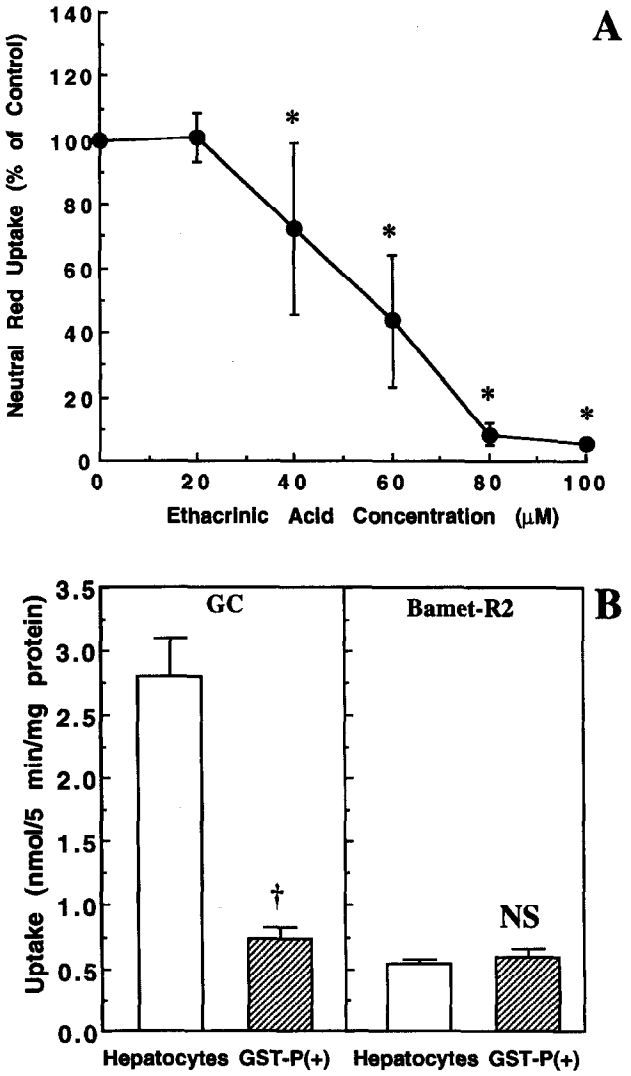

*Fig. 5. (A) Cell survival as measured by neutral red retention after treatment with ethacrinic acid at the indicated concentrations. Cells were isolated from rat livers at 20 weeks after the initiation of hepatocarcinogenesis. (B) Glycocholate (GC) and Bamet-R2 uptake over 5-min incubation (100 μM) by primary cultures of rat hepatocytes obtained from Control rats and pure population of GST-P(+) cells obtained by hepatocyte elimination with 100 μM ethacrinic acid of mixed cell populations isolated from rat livers at 20 weeks after the initiation of hepatocarcinogenesis. Values are means±S.E. of four experiments in which at least three culture dishes were measured. *p<0.05 as compared with 0.0 μM ethacrinic acid by paired t-test. †p<0.05, NS, p>0.05 as compared with hepatocytes by the Student t-test.*

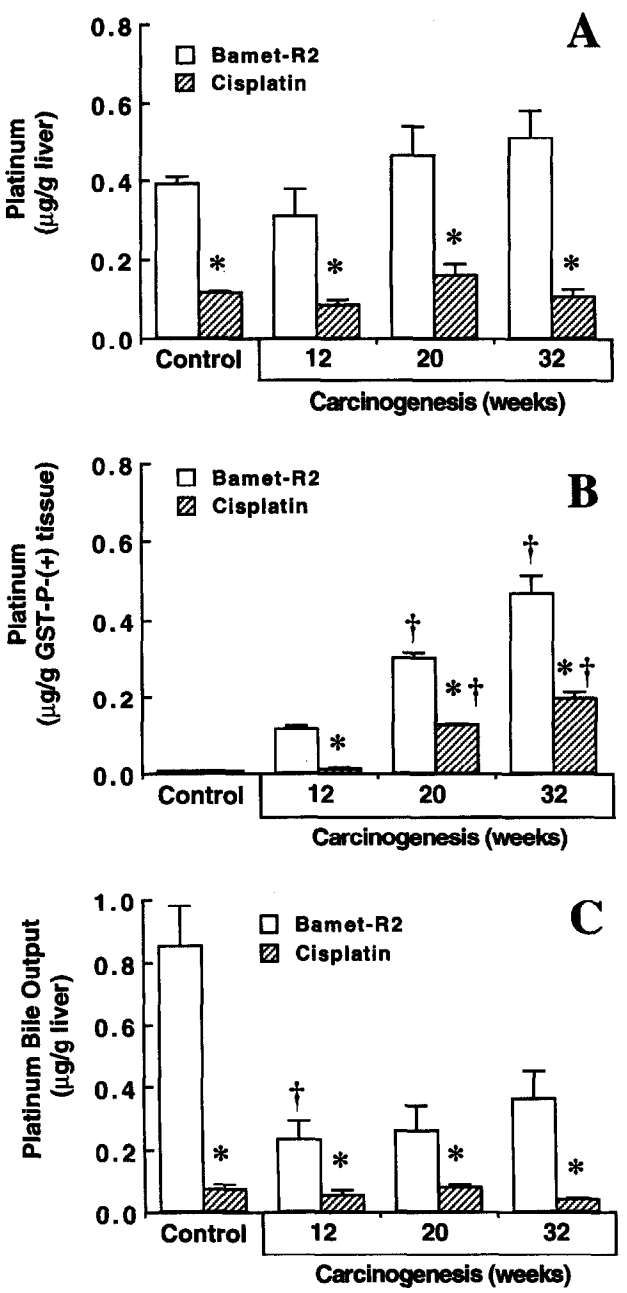

*Fig. 6. (A) Liver content of Bamet-R2 or cisplatin as measured by flameless atomic absorption of platinum after 15 min i.v. administration of 250 nmol Bamet-R2 or cisplatin in Control rats and at 12, 20 and 32 weeks after the initiation of hepatocarcinogenesis. (B) Calculated Bamet-R2 or cisplatin concentration in liver GST-P(+) tissue. (C) Bamet-R2 and cisplatin bile output during the 15 min after i.v. administration. Values are means±S.E. (in all groups n=5). *p<0.05 on comparing cisplatin to Bamet-R2; †p<0.05 as compared to the group on the left with similar treatment by the Bonferroni method of multiple range testing.*

at 20 weeks of carcinogenesis were isolated. Hepatocytes were eliminated by incubation for 48 h with ethacrinic acid. This treatment induced a marked dose-dependent reduction in the number of living cells (Fig. 5A). Although toxicity against both GST-P-positive and GST-P-negative cells was observed, ethacrinic acid was more efficient at killing hepatocytes than GST-P-

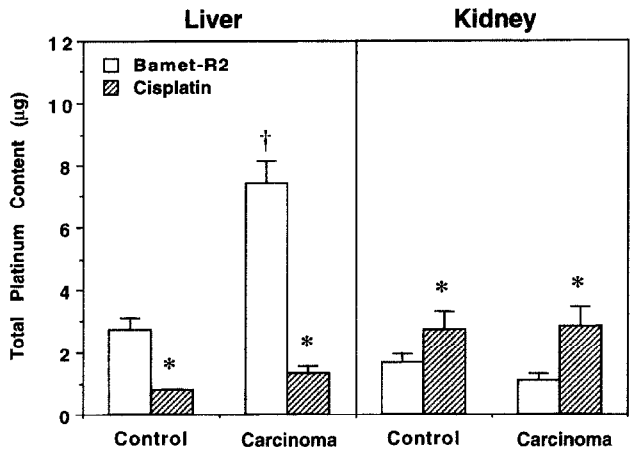

Fig. 7. Comparison of total liver and kidney contents of Bamet-R2 or cisplatin as measured by flameless atomic absorption of platinum after 15 min i.v. administration of 250 nmol Bamet-R2 or cisplatin to Control rats and liver carcinoma-bearing rats (32 weeks after the initiation of hepatocarcinogenesis). Values are means±S.E. (in all groups n= *5).* †$p<0.05$ *as compared to Controls;* *$p<0.05$ *on comparing Bamet-R2 to cisplatin by the Bonferroni method of multiple range testing.*

positive cells. Thus, after incubation with 100 μM ethacrinic acid only a small proportion of the initial cell population remained alive. Immunohistochemistry of these cells in culture (data not shown) revealed that almost all of them were small, round-shaped GST-P-positive cells. Uptake of GC and Bamet-R2 by these cells was compared with that of hepatocytes isolated from healthy rats (Fig. 5B). GC uptake was significantly reduced in GST-P-positive cells. However, no significant difference in Bamet-R2 uptake was observed.

When *in vivo* intravenous administration of Bamet-R2 or cisplatin to rats was rapidly (15 min) followed by determination of the amount of drug taken up by the liver and the kidney, no significant changes in the amount of Bamet-R2 or cisplatin retained by the liver tissue were observed during carcinogenesis (Fig. 6A). The amounts of Bamet-R2 or cisplatin retained by the neoplastic tissue were calculated on the assumption that the normal liver parenchyma had not modified its ability to take up these drugs (Fig. 6B). The results indicated that the amount of drug in GST-P-positive tissue was significantly increased during carcinogenesis. At all stages this was markedly higher for Bamet-R2 than for cisplatin. Cisplatin output into bile was low and not significantly affected by carcinogenesis (Fig. 6C). Bamet-R2 bile output was significantly higher than that of cisplatin. This was reduced from 12 weeks, but no further reduction during carcinogenesis was found. In all groups the amount of drug in the kidney was higher for cisplatin than for Bamet-R2 (Fig. 7). When the total amount of drug in the kidney was compared between the control and hepatocellular carcinoma-bearing rats (Fig. 7), no changes were found for cisplatin or Bamet-R2. By contrast, a slight and a marked increase in drug liver contents were observed for cisplatin and Bamet-R2, respectively.

Discussion

Recent investigations have reported the ability of human hepatocarcinoma cells to express both OATP and NTCP, which are involved in chlorambucil-taurocholate uptake (14). Results obtained in the present work support the hypothesis defended by Kullak-Ublick and co-workers on the feasibility of therapeutic strategies using antineoplastic agents coupled to bile acids with the aim of loading tumor cells with cytostatic drugs.

Cytosolic glutathione-S-transferases (EC 2.5.1.18) are a family of multifunctional enzymes that catalyse the nucleophilic addition of glutathione to electrophilic groups of a variety of compounds. This usually initiates a pathway that results in the elimination of potentially toxic substances. These enzymes are homo- and hetero-dimers of 12 different subunits encoded by four gene families: Alpha, Mu, Pi and Theta (31). In healthy rats the major glutathione-S-transferase (GST) in the liver parenchyma is formed by Alpha subunits. The isoenzyme Pi (GST-P), which is particularly abundant in placenta, is absent in rat hepatocytes but is expressed at detectable levels by biliary epithelium cells (32). By contrast, GST-P is expressed in neoplastic cells derived from liver parenchyma during carcinogenesis (33). Moreover, it has been recently reported that GST-P is a stable marker of preneoplastic and neoplastic cells throughout hepatocarcinogenesis in the rat (34). This characteristic was used in the present work to follow the evolution of initiated tissue after DEN-induced carcinogenesis. The time course of DEN-induced carcinogenesis was in agreement with previous works using 2-AAF and hepatectomy to stimulate promotion and progression (35). During the stage of focus formation, no histological separation between preneoplastic lesions, GST-P-positive and GST-P-negative tissue was found. As reported by others (36,37), our results indicate that part of this tissue underwent retrodifferentiation to normal liver parenchyma while another part of it evolved to encapsulated adenomas. Owing to the antitumor activity of the host immune system, some of these structures are eliminated, which may account for the reduction in the total proportion of GST-P-positive tissue observed at 32 weeks after DEN administration.

At this time, a marked atypia together with infiltration of fibrous capsules of some of the remaining nodules and invasion of the surrounding parenchyma were found. The liver-to-body weight ratio was maintained in all experimental groups, suggesting that a balanced growth between GST-P-positive tissue and normal parenchyma occurred during the 32-week experimental period. These are important observations for calculation of the amount of drug retained by the liver.

Although the expression of GST-P was maintained throughout carcinogenesis, it should be noted that the phenotypic characteristics of liver parenchyma are modified as part of the dysplasia leading to carcinoma formation. Thus, the ability of GST-P-positive tissue to secrete bile is progressively lost. This, together with the probable existence of intrahepatic obstructive cholestasis due to the obliteration of bile ducts by space-occupying, growing tumor tissue, would be responsible for the observed reduction in bile flow.

Bile acids are concentrated 10- to 50-fold in the hepatocytes, mainly via Na^+-dependent and Na^+-independent carrier-mediated systems (for review, see 1). FITC-GC is a fluorescent bile acid derivative that is efficiently taken up by hepatocytes (38) and secreted into bile (39). This has permitted its use as a cholephilic compound (39,40) that partly or wholly shares the natural mechanisms of bile acid transport from blood to bile across the hepatocyte. In the present work we observed that despite a marked reduction in FITC-GC output into bile following i.v. administration, this compound was taken up by most GST-P-positive cells at all stages of carcinogenesis. However, a certain amount of GST-P-positive tissue lacking this ability was found to co-exist in separate areas on the same liver, both at adenoma and carcinoma stages. This is an important observation, which suggests both that phenotypic changes related to dysplasia can lead to an inability to take up bile acids, and that this is not a common characteristic of liver tumors.

The liver organotropism of the cytostatic bile acid derivative Bamet-R2 has been reported previously (18). The facts that early on after administration the levels of Bamet-R2 in liver tissue were higher than those of cisplatin and that the opposite was found in kidney further support this concept. The results obtained in cultured GST-P-positive cells are preliminary findings but do provide clear evidence of the existence of efficient bile acid uptake by these cells. Lowered GC uptake together with unchanged Bamet-R2 uptake by GST-P-positive cells is consistent with differential effects of carcinogenesis on the expression and functionality of OATP and NTCP (14). Because Bamet-R2 is taken up mainly via OATP (18), the present results suggest that no marked alteration occurs in this transport system in GST-P-positive cells, while the activity of NTCP in these cells is probably reduced. Nevertheless, a complete kinetic and molecular characterization of these transport systems at different stages of carcinogenesis should be undertaken.

The absence of changes in the amount of drug found in the kidney during carcinogenesis contrasted with the observation of a progressive enhancement in the amount of platinum in liver GST-P-positive tissue. Bamet-R2 accumulation in neoplastic tissue was probably due to two different events: on one hand, the reduction in the excretory ability of this tissue and, on the other, the non-reversible formation of adducts with DNA in proliferating cells. When cisplatin, and presumably Bamet-R2, are dissolved in serum, where the Cl^- concentration is above 100 mM, the chloride ion belonging to the structure of these compounds remains bound to them. Once in the intracellular space, where the chloride concentration drops to approximately 3 mM, these compounds are activated by chloride displacement to allow the formation of aquo species, which are the reactive forms of this bioinorganic complex through their behavior as potent electrophiles. These react mainly with nucleophilic groups of nucleic acids, leading to the formation of DNA adducts, which are believed to account for the cytotoxic activity of this drug (17).

In sum, the present results indicate that at different stages of carcinogenesis, ranging from neoplastic lesions to carcinoma, nodules with the ability to take up bile acid derivatives co-exist with those lacking this characteristic. Thus, cytostatic drugs can be directed toward some types of liver tumors by coupling them to bile acids as long as the resulting compound is also taken up by tumor cells and retains part of the antiproliferative capacity of the parent drug. Both requirements are fulfilled by Bamet-R2.

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